REMARKS

Entry of the foregoing and favorable reconsideration of the subject application, as amended, pursuant to and consistent with 37 C.F.R. Section 1.112, and in light of the remarks which follow, are respectfully requested.

By the present amendment, the specification has been amended to provide the SEQ ID NO for the nucleotide sequence of Figure 2B (human nCL1 cDNA). Furthermore, by the present amendment, claims 1, 4, 5, 7, 8, 15 to 18 and 20 have been amended to further clarify the present invention. Claims 9 to 14 and 19, 21 and 22 have been cancelled since they are directed to a non-elected invention. Claim 6 has also been cancelled. However, Applicants reserve their rights to file a continuation or divisional application directed to this cancelled subject matter.

Another copy of the table of contents for *L'AND Médicament* (Axel Kahn ed., John Libbey Eurotext, 1993) is enclosed for the Examiner's consideration. The DNA sequences of the invention are not found in this book. Instead, page 5, lines 6 to 9, of the specification refers to *L'AND Médicament* describing vectors for gene therapy.

The disclosure has been objected to since the SEQ ID NO is missing from the paragraph 3 at page 6 of the present application. The specification has been amended to insert the SEQ ID NO corresponding to the nucleotide sequence. Therefore, this objection should be rendered moot by the above amendment of the specification.

Claims 15 to 17 have been rejected under 35 U.S.C. §112, first paragraph as lacking written description. This rejection has been obviated by amendment of claims 15 to 17; i.e., the recitation of the flanking sequences has been deleted these claims. However, Applicants reserve their rights to file a continuation or divisional application directed to this cancelled subject matter.

In view of the above, withdrawal of this rejection is respectfully requested.

Claim 20 has been rejected under 35 U.S.C. §112, first paragraph as lacking enablement. This rejection is respectfully traversed.

Claim 20 is directed to a pharmaceutical composition for treating LGMD2 disease which comprises a nucleic acid sequence coding for a calcium dependent protease enzyme or a host cell transformed with said nucleic acid sequence or an amino acid sequence encoded by said nucleic acid sequence.

In rendering this rejection, the Examiner purports that absent any teaching or guidance in the specification, the skilled practitioner in the art would be subjected to undue experimentation in attempting to practice the claimed pharmaceutical composition for the treatment of an LGMD2 disease.

Thus, the issue to be addressed with respect to this enablement rejection is whether the skilled artisan can practice Claim 20 of record without undue experimentation. The legal criteria for evaluating the undue experimentation issue are set forth in *In re Wands*, 858 F. 2d 731, 8 USPQ 2d 1400 (Fed. Cir. 1988) and are the following:

(1) the quantity of experimentation necessary;
(2) the amount of direction or guidance presented;
(3) the presence or absence of working examples;
(4) the nature of the invention;
(5) the state of the prior art;
(6) the relative skill of those in the art;
(7) the predictability or unpredictability of the art; and
(8) the breadth of the claims.

Although the Examiner has addressed points (2) and (3) above in the Official Action, and relies on Orkin et al. (of record) to deem that gene therapy is unpredictable, addressing point 7 above, the Examiner has failed to address the other points set forth in *In re Wands, supra*.

It should be said that the state of the prior art with respect to retroviral and adenoviral, as well as other constructs for gene therapy purposes was well established prior to the filing of the present application. This is evidenced by Orkin et

al. in Tables 1 and 2, which disclose the various vector systems which were currently in use or under consideration for gene therapy prior to December 1995. Table 2 of Orkin et al. discloses that 106 clinical trials involving gene transfer were underway in the U.S. alone. One-hundred and six clinical trials are a good indication that the state of the art, as well as the relative level of skill of those in this art, was very high at the time of filing of the present application.

Since the level of skill and the state of the art was at such a high level, the amount of direction and the presence or absence of working examples was not set forth in great detail in the present patent specification. Furthermore, it is not necessary to provide well known techniques as indicated in *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F2d 13107, 1384, 231USPQ 81.94 (Fed. Cir. 1983) *cert denied*, 480 U.S 94 (1987) where the Federal Court stated:

> A patent need not teach and preferably omits, what is well known in the art.

Furthermore, the breadth of Claim 20 is not that large that the skilled artisan cannot reproduce it. The specification provides enough guidance to obtain the isolated nucleic acid sequences of claim 1 and the isolated amino acid sequence of claim 5. These sequences are in fact set forth in the specification.

Moreover, a host cell transformed or transfected with the nucleic acid of claim 7 was easily obtained by the person skilled in the art at the time of filing this application as evidenced, for example in Orkin et al., Table 1, Annex I and Annex II. To make a vector construct and express a protein using nucleic acid sequences was simply common general knowledge and routine practice.

Thus, what remains in this evaluation is whether the art was predictable or unpredictable. Applicants submit that the art was not as unpredictable as the Examiner purports. The Examiner relies on Orkin et al. (1995) and states that this reference "reviewed the state of gene therapy art and reported that, among other problems, '[e]fficacy has not been established for any gene therapy protocol.'" However, since no efficient protocol has been established does not mean that gene

therapy is unpredictable. Indeed, the explanation given for this phenomenon in Orkin et al. is that the patients in the trials were previously treated with other medications, so it was difficult to measure the extent of gene therapy treatment.

Furthermore, the Examiner relies on the fact that Orkin et al. discloses "low frequency of gene delivery to target cells and the lack of definable biochemical or clinical endpoint." However, the disclosure of low frequency of delivery is irrelevant in the present case, since LGMD2 disease is a disease of the muscle and thus a person skilled in the art would contemplate directly injecting into muscle cells the nucleotide sequence, amino acid sequence or the host cells set forth in Claim 20.

Indeed, successful gene deliver was attained in muscle cells by direct injection as indicated in Annex I (Quantin et al.) and Annex II (Stratford-Perricaudet et al.). Thus, Quantin et al. demonstrate expression of a recombinant adenoviral vector in muscle cells. Stratford-Perricaudet et al. also demonstrate a long term gene transfer to mouse and skeletal muscles. Both of these articles had publication dates of 1991 and 1992, respectively; i.e., two and three years prior to the filing date of the present invention. Thus, Applicants submit that the unpredictability in the art was not high as the Examiner maintains.

The Examiner also mentions in the Official Action that there are no set dosages and no disclosed vector constructs for gene therapy set forth in the specification. However, relying on the state of the art for the previous clinical trials known in the art at the time of filing of the application, a skilled artisan would know what the general dose ranges were and repeat administration of the various dosages known in the art would be considered simply routine. Furthermore, the specification does refer to a book for guidance in the manner to make the vector construct. Also, the art at the time of filing the application discloses a variety of vector constructs that can be used in gene therapy, as evidenced by Quantin et al. and Stratford-Perricaudet et al.

The Examiner deems that the specification fails to identify any biochemical or clinical endpoints of the proposed gene therapy using the Claim 20 composition. However, pages 22 and 23 of the specification describe various methods that can be

used to detect LGMD2. A skilled artisan, once the therapy of injections has started could monitor the progress of the therapy, which is simply routine practice.

Hence, the quantity of experimentation that is necessary to obtain the vector construct used in the composition for gene therapy and the dosage would be a matter of routine and hence would not be considered undue experimentation.

Therefore, when evaluating undue experimentation according to *In re Wands, supra*, Applicants that the specification provides enough guidance to make the particular compositions set forth in Claim 20 and the prior art and relative skill of those in the art prior to filing the present application provides enough guidance such that Claim 20 is in fact enabled.

Finally, Applicants are enclosing herewith Annex 3, which demonstrates calpain 3 transfer in muscle using an AAV vector resulted in sustained calpain 3 expression with a localization of the protein. Please note that calpain 3 is described at least on page 4, lines 15 to 18, and forms a part of the present invention.

If the Examiner would like this data presented in the form of a Declaration, Applicants will gladly furnish one.

Thus, Applicants submit that the present invention is enabled for pharmaceutical composition for the treatment of an LGMD2 disease.

Therefore, in view of the above, withdrawal of this rejection is respectfully requested.

Claims 1 to 8 and 12 to 20 have been rejected under 35 U.S.C. §112, second paragraph as being indefinite. This rejection has been obviated by the amendments of claims 1 to 8, 15 to 18, and 20 according to the suggestions of the Examiner.

More specifically, as suggested by the Examiner, the terms "still" and "said" are no longer recited in claim 1. Furthermore, in claim 15, the term "such" has been replaced by --the--. In claims 16 and 18, the term "derived from" has been replaced

by --obtained from--. Claims 15 to 18 have been amended and no longer recite nonelected subject matter.

In view of the above, withdrawal of this rejection is respectfully traversed.

Claims 1 and 5 to 7 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Sorimachi et al. (J. Biol. Chem. 264: 20106-20111, 1989). This rejection has been obviated by the amendment of claim 1(c) and cancellation of claim 6.

In view of the above, withdrawal of the rejection pursuant to 35 USC § 102 (b) is respectfully requested.

From the foregoing, favorable action in the form of a Notice of Allowance is respectfully requested and such action is earnestly solicited.

Respectfully submitted,
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ANNEX I

Human Gene Transfer. Eds O. Cohen-Haguenauer, M. Boiron. Colloque INSERM/John Libbey Eurotext Ltd. © 1991, Vol. 219, pp. 271-278.

Adenovirus as an expression vector in muscle cells. Application to dystrophin

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SUMMARY

A recombinant adenovirus was constructed to target gene expression in muscle cells. Using β-galactosidase as a reporter gene, we were able to detect its expression in myotubes after infection of established cell lines and muscles of newborn mice.

INTRODUCTION

Adenovirus has been shown to be a candidate vector for gene therapy (1,2). We are investigating its potential for muscle diseases. Its advantages include a large host range (and thus the possibility to use the animal models available for Duchenne Muscular Dystrophy), the capacity of the vector for foreign DNA (at present 7 kbp, but in principle >30 kbp, thus compatible with the size of the dystrophin coding sequences), and a low pathogenicity in man.

METHODS

The recombinant virus was obtained as shown in Fig.1, and assayed on myogenic cell lines from mouse (C2.7) and rat (L6).
Newborn mice were infected by the intramuscular route; muscles were embedded in paraffin and sectioned after β-galactosidase detection (3).

RESULTS AND DISCUSSION

We have constructed a recombinant adenovirus where β-galactosidase is under the control of a mouse skeletal α-actin promoter reinforced by an enhancer from a mouse myosin light chain gene (MLC1-3F).
β-galactosidase expression was detected in infected myogenic cells and in mice muscle (Fig.2), but not in NIH3T3 fibroblasts. An expression was obtained even when fused myoblasts cultures were infected, suggesting that myotubes themselves can be infected.
The regulatory sequences we used should be suitable to direct muscle specific expression of a "minidystrophin" resembling that described in a family with mild Becker Muscular Dystrophy (4).

REFERENCES

1. Chasse J.F., et al.,(1989), Medecine/Sciences, 5, 331-337.
2. Stratford-Perricaudet L.D., et al., (1990), Human Gene Therapy, 1, 241-256.
3. England S.B., et al., (1990), Nature, 343, 180-182.
4. Sanes J.R., Rubenstein J.L.R., and Nicolas J.F., (1986), EMBO J., 5, 3133-3142.

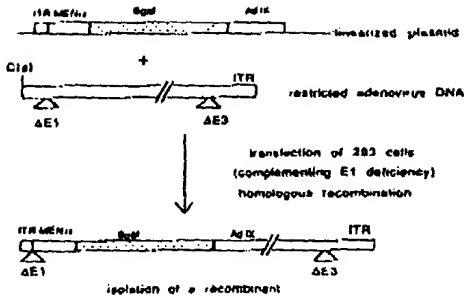

Fig.1: Obtention of a recombinant adenovirus.
MEN : enhancer of the mouse MLC1-3F gene
α : mouse skeletal α-actin promoter
βgal: β-galactosidase gene of *E.coli*
AdIX: sequences coding for peptide IX of adenovirus, with 3' flanking region
ITR : inverted terminal repeat.

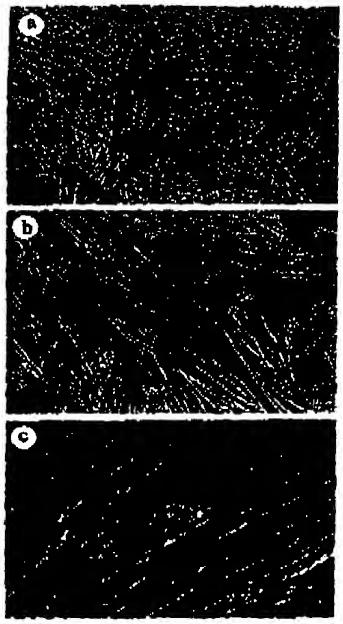

Fig.2: Expression of β-galactosidase following infection of:
a. C2.7 myotubes (24h infection)
b. L6 myotubes (24h infection)
c. newborn mice (12 days infection)

RESUME
Nous avons utilisé l'adenovirus comme vecteur d'expression dans les cellules musculaires. En utilisant le gène β-galactosidase comme "reporter", nous avons obtenu une expression après infection de myotubes de lignées myogéniques et de muscles de souris.

intravenous
intramuscularly

0. pp 2

Attachment D6

ANNEX II

Wid spread L ng-term G ne Transfer to M us Sk l tal Muscles and Heart

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Abstract

Successful treatment of muscular disorders awaits an adapted gene delivery protocol. The clinically applicable technique used for hematopoietic cells which is centered around implantation of retrovirally modified cells may not prove sufficient for a reversal of phenotype when muscle diseases are concerned. We report here efficient, long-term in vivo gene transfer throughout mouse skeletal and cardiac muscles after intravenous administration of a recombinant adenovirus. This simple, direct procedure raises the possibility that muscular degenerative diseases might one day be treatable by gene therapy. (*J. Clin. Invest.* 1992. 90:626–630.) Key words: adenovirus • gene therapy • β-galactosidase • muscular disease • eukaryotic viral vector

Introduction

The first genetic disorders amenable to gene transfer–based treatment will be monofactorial diseases. The vast array of target tissues translates the need for the development of appropriate, efficient gene transfer vehicles. The ability of retroviruses to integrate into the host genome has led to their use in ex vivo treatment protocols. Because those cell types capable of withstanding extraction, in vitro manipulation, and, finally, reimplantation are quite limited, other strategies need to be explored. Furthermore, the requirement retroviruses have for host cell proliferation constitutes an important drawback of such vectors and limits their applicability. Many targets relevant to human disease (liver, lung, muscle, neurons) will require other means of gene transfer.

Efficient and long-term expression of genes adenovirally transduced has recently been reported in hepatocytes and bronchial epithelium of animals (1–3), showing that the adenoviral vector is capable of transferring genes to nondividing or slowly proliferating cells. To investigate other potential targets for recombinant adenoviral vectors, we have constructed a recombinant adenovirus expressing a nuclearly targeted reporter enzyme (Ad.RSVβgal). The rapid in situ detection of the nuclearly targeted β-galactosidase allows an unambiguous and precise appreciation of adenoviral-mediated gene transfer (4, 5).

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J. Clin. Invest.

0021-9738/92/08/0626/05 $2.00
Volume 90, August 1992, 626–630

Methods

Construction of recombinant plasmid pAd.RSVβgal. The pAd.RSV-βgal is a pML-2 derivative where the nls lacZ gene with the SV40 early region polyadenylation signal (5, 6) driven by the Rous sarcoma virus long terminal repeat (RSV LTR)[1] is inserted downstream of 1.3 map units (mu) (PvuII site) from the left end of the adenovirus type 5 (Ad5) genome. The reporter gene is followed by mu 9.4–17 (BglII–HindIII fragment) of Ad5 to allow homologous recombination with the adenoviral genome for the generation of the recombinant adenovirus (Fig. 1).

Construction of recombinant adenovirus Ad.RSVβgal. The recombinant adenovirus was constructed by in vivo homologous recombination (7) in 293 cells (8) between plasmid pAd.RSVβgal and Ad dl327 (9) genomic DNA. Briefly, 293 cells were cotransfected with 5 μg of linearized pAd.RSVβgal and 5 μg of the large ClaI fragment (2.6–100 mu) of Ad5 DNA. After overlaying with agar and incubation for 10 d at 37°C, plaques containing recombinant adenovirus were picked and screened for nuclear β-galactosidase activity. The recombinant virus was propagated in 293 cells and purified by cesium chloride density centrifugation. Titers of the viral stocks were determined by plaque assay using 293 cells.

Injection of mice. 2- to 5-d-old and adult (2–3 mo) mice (C57BL6 × DBA) were injected either intravenously (iv) or intramuscularly (im) (quadriceps) with 20–40 μl of highly purified recombinant adenovirus Ad.RSVβgal (10^{11} plaque-forming units [pfu]/ml).

Escherichia coli β-galactosidase assay. Organs from killed animals were fixed in 4% *p*-formaldehyde in PBS for 30 min. After rinsing they were incubated overnight at 30°C in X-gal solution (2 mM) (6). Whole specimens were flash frozen in isopentane in liquid nitrogen and mounted in OCT compound (Miles Laboratories Inc., Naperville, IL) for cryosectioning. Sections (10 μm thick) were fixed 10 min in *p*-formaldehyde as described for organs, rinsed, and incubated with X-gal substrate. Sections were then counterstained with hematoxylin and eosin according to standard methods. Muscle was dissociated after whole organ staining to obtain isolated myofibers that were then counterstained with hematoxylin and eosin. Urine and fecal matter were collected at 2 h or 22 d after iv injection and exposed to 293 cells. After 24 h of incubation at 37°C the cells were fixed and stained with X-gal solution.

DNA analysis of animals. The heart, lung, liver, and quadriceps muscle from mice iv-injected with the recombinant adenovirus Ad.RSVβgal were minced into liquid N_2 and ground with a mortar and pestle. Total cellular DNA was prepared as described (10) and 10 μg of either undigested or HindIII-digested DNA was subjected to electrophoresis in a 0.8% agarose gel. Southern blot analyses (Fig. 4 *A*) were performed using either a SalI-BamHI fragment containing LacZ from pGEM-nlsLacZ (6), or a fragment from the pAd.RSVβgal plasmid containing the RSV promoter and the upstream adenovirus sequence (Fig. 4 *B*) to screen for the presence of the recombinant adenovirus.

1. *Abbreviations used in this paper:* Ad5, adenovirus type 5; DMD, Duchenne muscular dystrophy; mu, map units; pfu, plaque-forming units; RSV LTR, Rous sarcoma virus long terminal repeat.

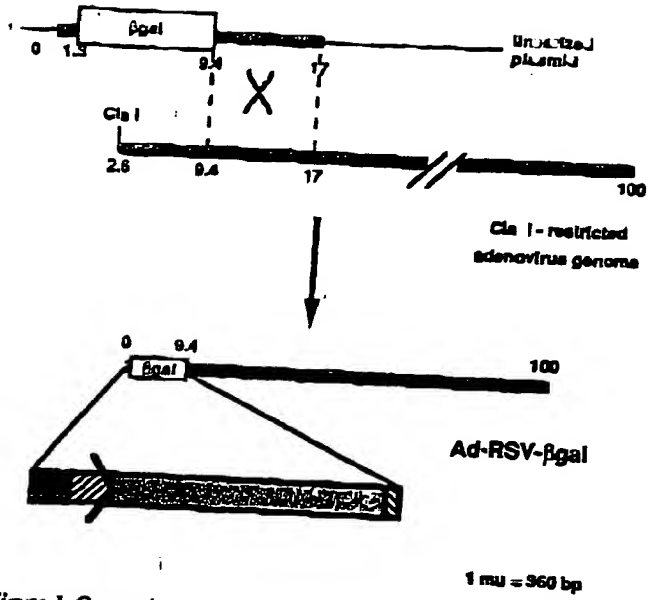

Figure 1. Generation of recombinant adenovirus, Ad.RSVβgal, by in vivo recombination. The recombinant adenovirus was constructed through homologous recombination between plasmid pAd.RSVβgal and the Ad5 genome. Shown also is an enlargement of the insert. The adenoviral sequences are depicted by ▬. The nlslacZ gene ▭ is controlled by the RSV LTR ▨ and possesses the early mRNA polyadenylation signal from SV40 ▬.

Results

Generation of the recombinant adenovirus, Ad.RSVβgal. Ad.RSVβgal is a recombinant adenovirus that constitutively expresses β-galactosidase targeted to the nuclei of infected cells. The nls lacZ coding sequence with the SV40 early region 3'-end processing signal and under the control of the RSV LTR was inserted into Ad dl327 in place of E1a and E1b (mu 1.3–9.4). Fig. 1 depicts the construction of the recombinant virus. Plaques resulting from the transfection of 293 cells were screened for β-galactosidase activity. Virus was amplified on 293 cells. The recombinant virus is replication incompetent due to its deletion for the E1 genes.

Expression of the transferred gene in mice injected as neonates. Neonatal mice were intravenously injected with the Ad.RSVβgal recombinant virus, and gene transfer was assessed by histochemical staining for β-galactosidase activity in various tissues. The extent of blue staining reveals that a substantial percentage of cells within different tissues are infected. Positive perinuclear staining was systematically observed in many organs such as lung, liver, intestine, heart, and skeletal muscle (Fig. 2, *A–F*) of each of the four individual mice killed at 15 d after iv injection.

The exciting implications of efficient gene transfer into myocytes led us to characterize transduction to these cells in particular. Gross examination of the intact heart as well as skeletal muscles from the experimental animals reveals the impressive efficiency of gene transfer after only a single injection of the recombinant adenovirus (Fig. 2, *C, D,* and *F*). Because the intravenous route was used, the viral vector is not concentrated in any one area of the muscle tissue, and dispersion is favored. Histochemical staining of muscle leads to large patches of blue throughout. Approximately 0.2% of cardiac cells have undergone gene transfer after iv injection of 10^9 pfu of virus.

Expression of the transferred gen in both cardiac and skeletal muscle was found to be remarkably stable since monthly killed injected animals displayed β-galactosidase activity in these tissues throughout the 12-mo period of the experiment. Importantly, gene expressi n was sustained, although the proportion of blue cells in these muscle tissues seemed to decrease after the 10th month after injection.

Analysis of isolated fibers demonstrates the extent of dissemination of the transferred gene. A single fiber can show multiple "centers of expression" (Fig. 3). Each consists of a darkly stained central nucleus surrounded by nuclei forming a gradient of blue. These centers most probably result from independent local infections by the adenoviral vector. The number of colored nuclei in any one cluster was found to vary from 10 to 60. The use of a nuclearly targeted marker is informative as to the degree of infection of multinucleated cells of this morphology.

Expression of the transferred gene in mice injected as adults. As to the distribution of the virus, results similar to those obtained in mice injected as newborns were found after intravenous inoculation of adults. It is apparent, however, that the injection of 10^9 pfu of virus leads to less efficient gene transfer in the larger adult mice. Intramuscular injections were also performed and histochemical staining at 21 days after injection revealed that the infection was circumscribed to the point of injection, as fibers with blue nuclei could only be detected within a 1-cm area. Moreover, the absence of blue staining in other tissues (lung, liver, intestine) reveals a very limited diffusion of the virus when the intramuscular route is chosen.

Status of the viral DNA. Southern blot analysis of DNA from different tissues of an experimental animal indicates the presence of the adenovirus genome in a wide variety of organs (Fig. 4 *A*). The detection of a DNA fragment corresponding to the left end of the recombinant viral genome indicates that the viral DNA is present as a linear form in the tissues infected 10 d previously (Fig. 4 *B*). Moreover, the detection of a unique and intense band corresponding to 35.5 kb at 3 mo after injection reveals that the viral DNA remains extrachromosomal (Fig. 5). The absence of a detectable smear rules out the possibility that integration events occurred with high frequency. Clearly, the sensitivity of the Southern blot does not allow a fair appreciation of gene transfer as does the in situ detection of the β-galactosidase activity. In this respect, it is reasonable that the estimation of a 0.2% transfer to the heart (based on staining) is compatible with the difference in intensity seen with the transgenic mouse DNA control (which harbors one copy of a LacZ gene per cell) (Fig. 4 *A*). The incapacity to detect a band in the heart 12 mo after injection is in agreement with the decrease in the number of blue cells observed at this time.

Discussion

Gene therapy relevant to muscular diseases is especially hindered by the unsolved problem of the direct widespread transfer of a gene to the related tissues. Attempts to modify muscle tissue have centered around fusion of implanted myocytes with host muscle (11, 12) or injection of DNA (13–15). Fusion in mice of normal donor muscle precursor cells with host mdx myofibers (11) has borne excitement leading to preliminary trials of such cell therapy in children. This approach

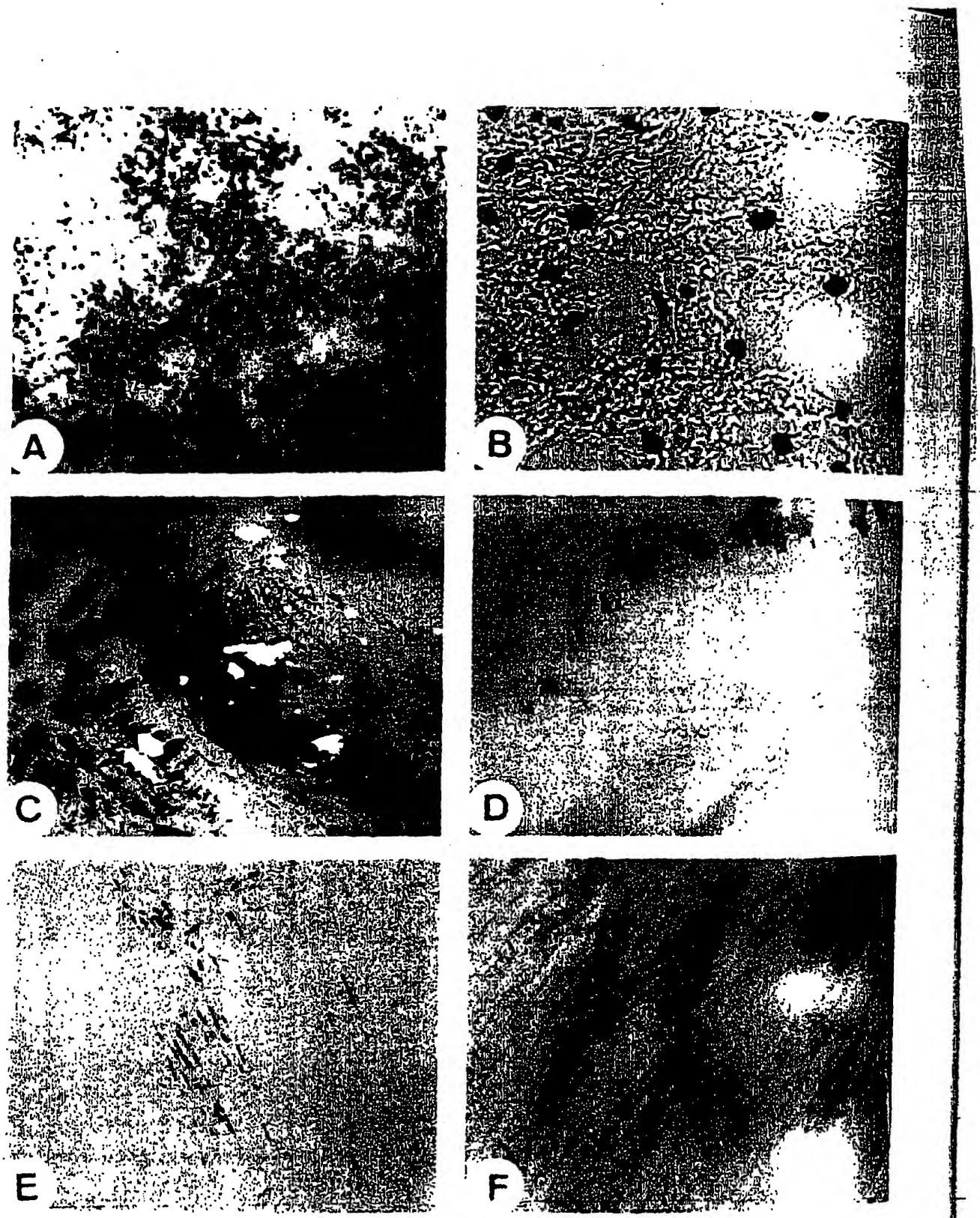

Figure 2. Efficient adenoviral-mediated gene transfer to mouse organs as evidenced by histochemical staining for β-galactosidase activity. (*A*) Liver (×40); (*B*) lung (×400); (*C*) heart atrium (×40); (*D*) heart ventricle (×40); (*E*) intestine (×40); (*F*) skeletal muscle (×40). Newborn mice were injected intravenously and organs were removed 15 d after injection for in situ assay for β-galactosidase activity.

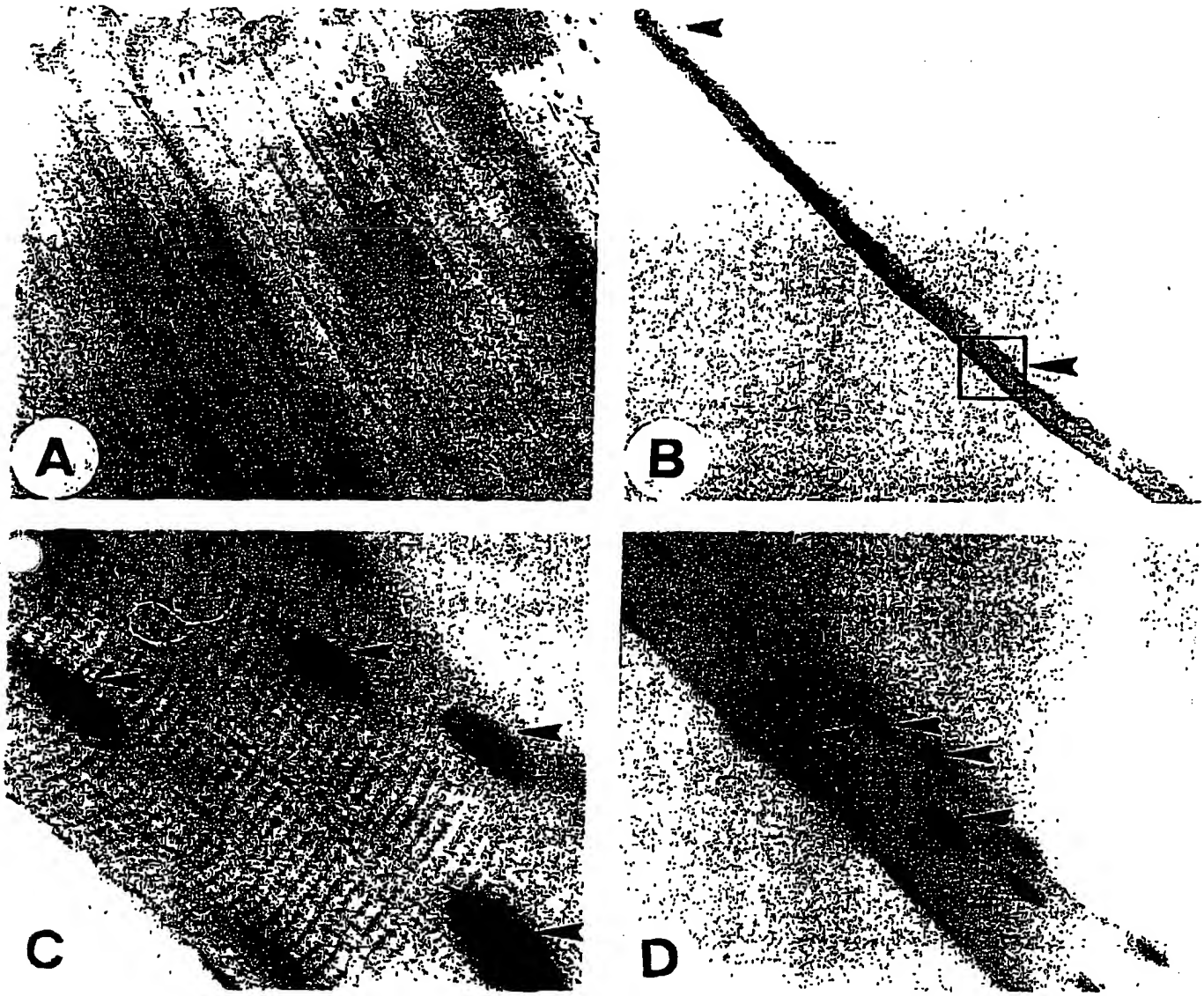

Figure 3. Detailed analysis of gene transfer into skeletal muscle after iv injection of Ad.RSVβgal. Gene expression was assessed 5 mo after injection by histochemical staining for nuclear β-galactosidase activity. (*A*) Dorsal skeletal muscle (×40); (*B*) isolated dorsal muscle fiber (×40). Arrowheads indicate two centers of expression. (*D*) Enlargement of boxed area in *B* (×250). A dark blue source nucleus is surrounded by nuclei of varying intensity of blue staining. (*C*) Enlargement of center of expression shown in *D* (×500). Arrowheads indicate source nucleus and nuclei of immediate vicinity.

may, nevertheless, have too many drawbacks for it to be applicable to the treatment of disease. As the migratory capacities of precursor cells are restricted to a few millimeters, cell implantation would necessitate millions of injections during hours of anesthesia. Inevitably, immunological problems would be encountered, as with any graft. Furthermore, large scale requirements for human myogenic cells constitute a practical limitation. In addition, the treatment of Duchenne muscular dystrophy (DMD) not only calls for therapy for skeletal muscles, but for myocardial cells too. It is difficult to envisage cell therapy as a means to provide relief to such an array of diseased cells.

The concept of somatic gene therapy will more than likely provide the most promising solution in the future. Importantly, its scope goes beyond the treatment of muscle disease, since it is applicable to a large number of genetic disorders. The direct introduction of purified nucleic acids into various organs in vivo is attractive due to its simplicity, but again, practical obstacles may limit its development. Furthermore, the resultant gene expression in muscle remains localized to the point of injection of DNA (13) and seems to be quite limited in duration, particularly in cardiac muscle (14). Interestingly, all other organs tested proved to be nonreceptive to DNA transfection. Thus, a method allowing a more widespread distribution of stable gene expression would be of invaluable importance to gene therapy in general.

The present report demonstrating the feasibility of adenovirus-mediated direct in vivo gene transfer into myocytes of mice has serious implications for treatment of muscular disorders, including heart diseases. The proportion of skeletal and myocardial cells expressing th transferred gene is more in keeping with that probably required for a reversal of disease state. It is

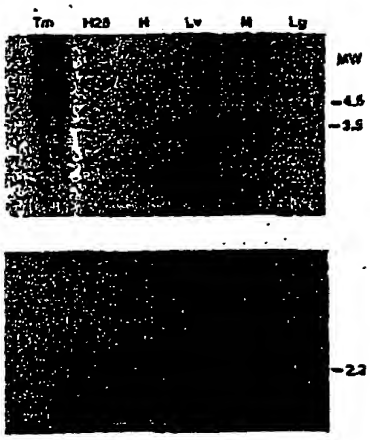

Figure 4. Southern blot analyses. All lanes contain 10 μg of HindIII-digested tissue DNA hybridized either with a Lac Z probe (*A*) or with a probe specific for the left end of the recombinant virus (*B*). Tm corresponds to the DNA of a transgenic mouse which contains one copy/cell of a Lac Z gene. DNA from the heart (*H*), liver (*Lv*), skeletal muscle (*M*), and lung (*Lg*) of a mouse killed at 10 d after injection were analyzed. H28 corresponds to heart DNA from a mouse killed 12 mo pi. Size markers (*MW*) are shown in kilobase-pairs in the right margin.

expected that the percentage of recipient cells be a function of the quantity of injected virus per animal weight. The possibility to obtain very high titers of adenovirus makes it conceivable to increase the input of virus for larger animals. The remarkable stability of expression observed (at least 12 mo), notwithstanding the extrachromosomal state of the vector, would be of safe therapeutic value.

An important distribution of the putative therapeutic DNA is a prerequisite for the treatment of muscular diseases like DMD. This can clearly be achieved when a recombinant adenovirus is administered intravenously since in this case dispersion of the vector occurs throughout the animal. In contrast, the direct intramuscular injection of a recombinant adenovirus can only lead to a localized gene transfer. It is noteworthy that gene transfer is successful not only in neonatal animals, but also in adults (albeit to a lesser extent), thus opening the route to gene therapy of diseases clinically diagnosed later in life.

Importantly, adenovirus can carry tissue-specific promoters, consequently restricting the actual sites of expression of the exogenous gene (16, 17). Moreover, the important cloning capacity of the adenovirus vector makes realistic and promising the construction of a recombinant adenovirus harboring the dystrophin gene. The recent report showing that expression of dystrophin can correct one of the effects of dystrophin deficiency (15), can only stress the urgency of an adapted vector. Taken together, the potentials of adenovirus along with its proven capacities, render this virus a most interesting gene delivery system for the treatment of the important hereditary human disease, DMD. The construction of an adenovirus harboring the dystrophin cDNA is now in progress in the laboratory. If such a virus is to be used in humans, safety aspects should be addressed concerning viral dissemination in the local environment. In this regard, it is noteworthy that no Ad.RSVβgal virus could be detected in urine and fecal matter after intravenous inoculation of the recombinant adenovirus even though a nuclear Lac Z expression could be detected not only in the smooth muscle of the intestine (Fig. 2 *E*), but also occasionally in a few epithelial cells of this organ (data not shown).

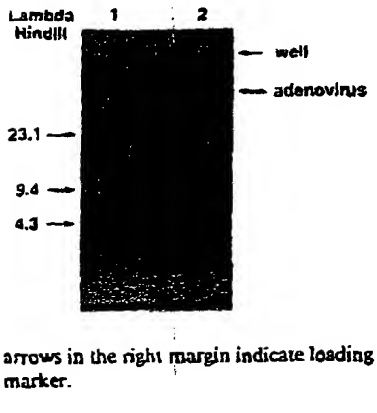

Figure 5. Southern blot analysis. Lane *1*, 10 μg of undigested tissue DNA prepared from a control heart; lane *2*, 10 μg of undigested tissue DNA prepared from the heart of an experimental mouse injected intravenously 3 mo previously. Size markers (Lambda/HindIII) are shown in kilobasepairs in the left margin, and arrows in the right margin indicate loading wells and adenovirus size marker.

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References

1. Stratford-Perricaudet, L. D., M. Levrero, J.-F. Chasse, M. Perricaudet, and P. Briand. 1990. Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Hum. Gene Ther.* 1:241–256.

2. Rosenfeld, M. A., W. Siegfried, K. Yoshimura, K. Yoneyama, M. Fukayama, L. E. Stier, P. K. Paakko, P. Gilardi, L. D. Stratford-Perricaudet, M. Perricaudet, et al. 1991. Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium in vivo. *Science (Wash. DC).* 252:431–434.

3. Rosenfeld, M. A., K. Yoshimura, B. Trapnell, K. Yoneyama, E. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L. Stier, L. D. Stratford-Perricaudet, et al. 1992. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 68:143–155.

4. Ralston, E., and Z. W. Hall. 1989. Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. *Science (Wash. DC).* 244:1066–1069.

5. Kalderon, D., B. L. Roberts, W. D. Richardson, and A. E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499–509.

6. Bonnerot, C., D. Rocancourt, P. Briand, G. Grimber, and J.-F. Nicolas. 1987. A β-galactosidase hybrid protein targeted to nuclei as a marker for developmental studies. *Proc. Natl. Acad. Sci. USA.* 84:6795–6799.

7. Gluzman, Y., H. Reichl, and D. Solnick. 1982. Helper-free adenovirus type 5 vectors. *In* Eukaryotic Viral Vectors. Y. Gluzman, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 187–192.

8. Graham, F. L., J. Smiley, W. C. Russel, and R. Nairu. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59–72.

9. Shenk, T., and J. Williams. 1984. Genetic analysis of adenoviruses. *Curr. Top. Microbiol. Immunol.* 111:1–39.

10. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

11. Partridge, T. A., J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel. 1989. Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature (Lond.).* 337:176–179.

12. Salminen, A., H. F. Elson, L. A. Mickley, A. T. Fojo, and M. M. Gottesman. 1991. Implantation of recombinant rat myocytes into adult skeletal muscle: a potential gene therapy. *Hum. Gene Ther.* 2:15–26.

13. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. 1991. Direct gene transfer into mouse muscle in vivo. *Science (Wash. DC).* 247:1465–1468.

14. Acsadi, G., S. Jiao, A. Jani, D. Duke, P. Williams, W. Chong, and J. A. Wolff. 1991. Direct gene transfer and expression into rat heart in vivo. *New Biol.* 3:71–81.

15. Acsadi, G., G. Dickson, D. R. Love, A. Jani, F. S. Walsh, A. Gurusinghe, J. A. Wolff, and K. E. Davies. 1991. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. *Nature (Lond.).* 352:815–818.

16. Friedman, J. M., L. E. Babiss, D. F. Clayton, and J. E. Darnell, Jr. 1986. Cellular promotein incorporated into the adenovirus genome: cell specificity of albumin and immunoglobulin expression. *Mol. Cell. Biol.* 6:3791–3797.

17. Karlsson, S., K. van Doren, S. Schweiger, A. Nienhuis, and Y. Gluzman. 1986. Stable gene transfer and tissue-specific expression of a human globin gene using adenoviral vectors. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2377–2385.

ANNEX III

Data supporting the possibility to use calpain 3 gene transfer for gene therapy of calpainopathies (LGMD2A)

Introduction

Calpainopathy (LGMD2A for limb girdle muscular dystrophy type 2A) is a recessive muscular disorder caused by deficiency in calpain 3, the muscle specific member of calpain family. At present, no treatment has been proved to be effective for this disease. In the murine model for LGMD2A, we evaluated the potential of intramuscularly delivered adeno-associated virus (AAV) for gene therapy.. rAAV vectors carrying the calpain 3 cDNA under the influence of a synthetic muscle-specific promoters were constructed. *In vivo* transfer efficiency was monitored by RT-PCR quantitative analysis of transgene expression and immunohistochemistry detection of the transfered protein. AAV vector administration resulted in sustained calpain 3 expression with a correct localization.

Methods

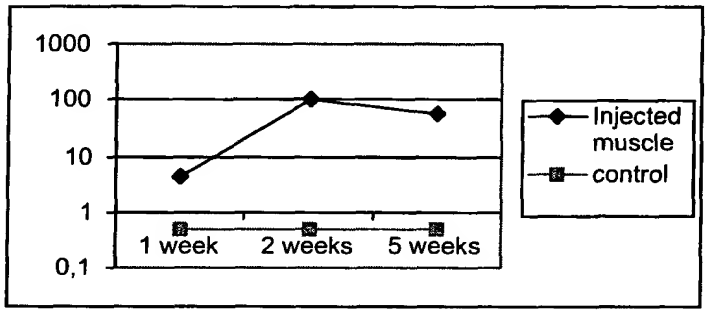
The complete coding sequence of murine Calpain 3 was obtained by restriction from a previously constructed plasmid and cloned under the influence of a synthetic muscle specific promoter into a cis-acting AAV plasmid. 293 cells were transfected with this vector and AAV helper plasmids containing the rep and cap genes. Cell lysates were harvested 72 h following transfection and purified by cesium chloride gradient centrifugation followed by dialysis. Physical and infectious particle levels were determined by the dot blot method and RCA test, respectively. Normal 129SV and calpain-deficient mice were injected with the AAV preparation in 20 µl of saline serum into the tibialis anterior muscle. The AAV and controlateral muscles were isolated and frozen in liquid nitrogen-cooled isopentane. Total RNA was extracted from muscles by Trizol method. Determination of transferred calpain 3 mRNA level was performed by real time PCR using specific primer pairs and probes. For the immunohistochemical study, 10 µM transverse or longitudinal cryosections from frozen muscles were incubated with 1/200 dilutions of calpain 3 primary antibodies Revelation was performed using a goat anti-rabbit secondary antibody diluted 1/100, and a tertiary donkey anti-goat antibody conjugated with a fluorogenic dye diluted 1/1000. Sections were visualized on a confocal microscope.

Results

Real-time RT-PCR analysis of calpain 3 expression.

A rAAV carrying the calpain 3 cDNA under the influence of a muscle-specific promoter was directly injected into muscle of calpain 3 deficient mice. Muscles were sampled at 1, 2 and 5 weeks. After isolation of RNA, evaluation of the level of mRNA expressed by the injected vector was performed by specific real-time RT-PCR. Messenger copy number was determined by comparison to a standard curve obtained using serial dilutions of a control plasmid. Expression was determined to reach a level of approximatively 100 copies per cell 2 weeks after injection. This is to be compared to the natural level of calpain 3 in muscle, which was previously determined to be between 100 and 1000 copies per cell.

Figure 1: Copy number of messenger expressed by a rAAV calpain 3 vector 1, 2 or 5 weeks after injection.



Immunodetection of calpain 3 protein in longitudinal section of injected muscle

Sections of injected muscles of calpain 3 deficient mice were analyzed by immunohistochemistry for detection of transferred calpain 3 protein. A staining that was clearly of sarcomeric pattern in some fibers was visualized. As the calpain 3 protein due to its binding to titin, the giant protein of the sarcomere was previously detected at the same location, this result suggests that the transferred protein is correctly localized.

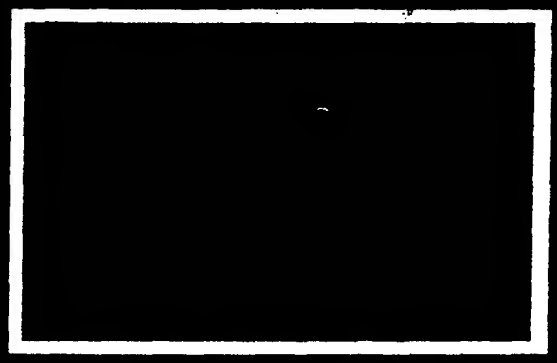
Figure 2: Detection of calpain 3 protein (in blue) in a muscle 5 weeks after injection.

Conclusion

Calpain 3 transfer in muscle using AAV vector resulted in sustained calpain 3 expression with a correct localization of the protein. These results support the idea that it is possible to use calpain 3 transfer as a therapeutic strategy.

m/s

coordonné par Axel Kahn



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